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Physiological differences between female limited, alternative life history strategies: the Alba phenotype in the butterfly *Colias croceus*

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Abstract

Across a wide range of taxa, individuals within populations exhibit alternative life history strategies (ALHS) where their phenotypes dramatically differ due to divergent investments in growth, reproduction and survivorship, with the resulting trade-offs directly impacting Darwinian fitness. Though the maintenance of ALHS within populations is fairly well understood, little is known regarding the physiological mechanisms that underlie ALHS and how environmental conditions can affect the evolution and expression of these phenotypes. One such ALHS, known as Alba, exists within female of many species in the butterfly genus *Colias*. Previous works in New World species not only found that female morphs differ in their wing color due to a reallocation of resources away from the synthesis of wing pigments to other areas of development, but also that temperature played an important role in these trade-offs. Here we build on previous work conducted in New World species by measuring life history traits and conducting lipidomics on individuals reared at hot and cold temperatures in the Old World species *Colias croceus*. Results suggest that the fitness of Alba and orange morphs likely varies with rearing temperature, where Alba females have higher fitness in cold conditions and orange in warm. Additionally shared traits between Old and New World species suggest the Alba mechanism is likely conserved across the genus. Finally, in the cold treatment we observe an intermediate yellow morph that may have decreased fitness due to slower larval development. This cost may manifest as disruptive selection in the field, thereby favoring the maintenance of the two discrete morphs. Taken together these results add insights into the evolution of, and the selection on, the Alba ALHS.

Key words: Life history traits, color morphs, respirometry, lipidomics, growth rate, *Colias*, Alba

Introduction

Life history strategies are co-evolved life history traits that effect the timing and patterns of development and reproduction (Stearns, 1992). Across a diverse range of taxa individuals within a single species, and even a single sex, can exhibit descrete alternative life history strategies (ALHS) wherein individuals differ in their behavior, size, color, morphology, or physiology due to trade-offs associated with divergent investment of resources (e.g. Lank *et al.*, 1995; Gross, 1996; Andres & Cordero, 1999; Tsubaki, 2003; Küpper *et al.*, 2015; Lamichhaney *et al.*, 2015). The maintenance of discrete alternative strategies within a population is intriguing, since such a situation is expected to be unstable, with the phenotype having the highest fitness expected to fix within populations, thereby removing the phenotypic variation (Fisher, 1930). Theoretical modeling has contributed to our understanding of how multiple morphs are maintained within populations, putatively due to balancing selection (Hofbauer and Sigmund, 2003). However questions still remain regarding the selection dynamics acting on, and the evolution of, ALHS. For example, is the repeated presence of an ALHS across related species the result of a conserved mechanism or have species converged on the same phenotype via different mechanisms? If the mechanism is conserved, in which environments does selection favor loss versus the maintenance of polymorphism? To gain insights to these questions we focus on Alba, a female-limited ALHS in butterfly species within the genus *Colias.*

Colias butterflies are widespread and can be found on every continent except Australia and Antarctica. About 1/3 of the approximately 90 species exhibit polymorphic females where Alba females have white wings and the non-Alba morph is yellow/orange similar to their conspecific males (Remington, 1954; Limeri & Morehouse, 2015). Previous work in the North American species *C. eurytheme* found Alba's white color is due to a large reduction in the amount of pteridine pigments in the wings. For example, Alba females exhibit a 20-fold reduction in sepiapterin (orange), a 7-8 fold in xanthopterin (yellow), and a 3-fold in erythropterin (red) compared to orange females. Meanwhile Alba females only exhibit a slight increase in the colorless pteridine, leucopterin (Watt, 1973). This massive reduction in the synthesis of colored pteridines allows Alba females in *C. eurytheme* to reallocate resources, specifically ~0.4 mg of GTP, which is several percent of the nitrogen budget, to other areas of growth and development (Watt, 1973). Presumably due to this trade-off Alba gains fitness advantages over orange

females. For example, Alba females in *C. scudderi* and *C. alexandra* have larger fat bodies (Graham *et al.*, 1980), a strong predictor of butterfly fecundity (Boggs, 1981). However, males (*C. alexandra* and *C. scudderi*) make their initial decision to court a female based on wing color (Graham *et al.*, 1980). As a result, Alba females are discriminated against during mate selection (Graham *et al.*,1980; Nielsen & Watt, 2000). Male discrimination potentially has a dramatic effect on Alba fecundity not only because it delays reproductive onset, but also delayed and less frequent mating will cause Alba females to lose out on the nutrient-rich spermatophores that males transfer to females upon mating. This may have significant adverse effects on fecundity and longevity (Graham *et al.*, 1980; Boggs, 1981). Additionally in *C. eurytheme* Alba benefits are temperature-dependent, for example in cold temperatures Alba females have significantly faster pupal development (4% faster than orange) and more mature eggs at eclosion (Graham *et al.*, 1980). Genetic crossing studies from six *Colias* species indicate that Alba is a single autosomal locus with Mendelian inheritance, where Alba is dominant (*AA, Aa*) (Remington, 1954). These results suggest that the Alba locus is conserved across the genus and thus far in the literature it has largely been treated as such. However, since the genetic mechanism remains unknown, it is also possible that the Alba phenotype evolved multiple times via different mechanisms. Therefore whether the trade-offs exist genus-wide remains an open question as previous investigations focused on New World species.

In this study we focus on *Colias croceus* (Geoffroy, Lepidoptera:Pieridae) (Figure 1), an Old World species widespread across Europe and North Africa. We provide insights into ALHS evolution by testing if Alba associated trade-offs are conserved between Old and New World species. Also we investigate potential genotype x environment interactions that may contribute to the evolution and/or maintenance of this ALHS by rearing butterflies at two temperature treatments and quantifying differences in major life-history traits (growth rate, developmental budget, adult weight, abdomen to thorax ratio and adult lipid store size) between the two strategies (Alba and orange). We generally hypothesize that the resources Alba females reallocate from wing pigment biosynthesis will result in Alba having faster or less energetically expensive development, or larger lipid stores as adults.

Methods

Butterfly husbandry, sample collection, and experimental design for measurements of life history traits

Six *Colias croceus* Alba females were collected from field sites in Catalonia, Spain (Sant Antoni de Vilamajor and Aiguamolls de l'Empordà Nature Reserve) in July 2015, and subsequently oviposited on *Medicago sativa* (Linnaeus, Fabales:Fabaceae) in the laboratory at Stockholm University. Eggs were moved into individual plastic rearing cups covered with netting and randomly split between two temperature treatments (Hot: constant temperature of 27°C, relative humidity ~ 54% for both larval and pupal development. Cold: constant temperature of 22°C during larval development and a constant temperature of 15°C during pupal, relative humidity ~ 43%. A 16-hour day length was used in both treatments and both developmental stages). Two different temperatures were used in the cold treatment because we were concerned that if reared at 15°C during larval development the growth period would extend beyond the growing season of the host plant. All individuals were visually checked at least every 12 hours throughout the entirety of development. Hatch time from the egg, pupation, and eclosion were recorded (in hours). Larvae were reared on *M. sativa* collected from the area surrounding Stockholm University; it was provided *ad libum* throughout development. Upon eclosion adults were stored at 4°C until the next day. The next day the wet weights of the abdomen and thorax were recorded and the abdomen to thorax ratio calculated. Removed abdomens were stored at -80°C for about one month before high performance thin layer chromatography (HPTLC) of lipids was performed. Individuals that had not excreted their meconium were not sampled. These experiments allowed us to test for differences among color morphs and cold and hot treatments in the following life-history traits: larval and pupal development rate, adult wet weight, and abdomen to thorax ratio.

Dynamic injection respirometry and metabolic rate measures

Previous studies found Alba females develop faster than orange during pupation (Graham *et al.*, 1980). To assess potential mechanisms underlying a development rate advantage we measured carbon dioxide (CO_2) production and oxygen (O_2) consumption of individual pupae of both sexes and morphs for both the hot and cold treatment daily throughout pupal development. To conduct these measurements individuals were weighed to the nearest 0.0001 g using a Sauter RE1614

scale and moved into 20 ml syringes that served as respirometry chambers on the second day of pupation and remained in the syringe throughout pupation. Delayed transfer on the second day of pupation allowed the outer cuticle of the pupa to harden which reduced the risk of damage during handling. Syringes were filled with ambient air that had been passed through filtered acidified water (pH < 4.5, checked weekly). Then the syringes were sealed with a three-way luer valve and kept at the respective temperature treatment (27°C or 15°C) for roughly 24 hours, except for during the daily respirometry measurement when they were at room temperature (~5-10 minutes per day) (for daily sample sizes see Table S1). At the start of each 24-hour measurement cycle the O_2 concentration in the syringe was ~21% and the humidity was ~35%. An empty syringe served as the control. The CO_2 production was measured with a Sable Systems (Las Vegas, USA) differential respirometry setup, as described previously (Lehmann *et al*., 2016). $\dot{V}CO_2$, $\dot{V}O_2$, respiratory quotient (RQ) and finally metabolic rate (MR) were calculated as previously described (Lehmann *et al.*, 2016). The RQ, sometimes called the respiratory exchange ratio, is the volume of carbon dioxide given off by tissues divided by the volume of oxygen absorbed by them (Schmidt-Nielsen, 1990) and indicates what substrate is being catabolized as an energy source. When carbohydrates are the sole energy substrate, RQ is ~1, when proteins ~0.8, and when lipids ~0.7 (Schmidt-Nielsen, 1990). Finally, the pupal developmental budget (DB) was calculated by converting MR (in watts) to daily energy expenditure (in joules) [daily energy expenditure = MR × 86400 (seconds per day)]. Then the daily energy expenditures for days 2-22 (the period that we had measurements across all individuals) were summed for each individual. DB was only calculated for the cold treatment.

Neutral lipid quantification by high performance thin layer chromatography (HPTLC)

HPTLC was used to quantify the main classes of neutral lipids within abdomens of 32 females (21 Alba and 11 orange) and 11 males from the cold treatment, and 25 females (14 Alba and 11 orange) and 12 males from the hot treatment. Total lipids were first extracted using the Folch method (Folch et al. 1957). Whole frozen *C. croceus* abdomens were homogenized in a mixture containing 50 µl milli-Q water, 320 µl methanol and 635 µl chloroform in Eppendorf tubes in a TissueLyser Bead Homogenizer (Quiagen, Hilden, Germany). The homogenate was transferred to 10 ml kimax tubes and 4.5 ml chloroform:methanol (2:1) was added. Tubes were vortexed vigorously for 10 sec, left at 23°C for 30 minutes in darkness, and centrifuged for 10 min at 3000

rpm. The upper phase was moved to a new kimax tube and 0.9 ml of water purified using ion-exchange was added. Following vigorous vortexing (10 seconds), tubes were again centrifuged for 10 min at 3000 rpm and the lower phase was moved to a new tube. This step was repeated on the original tube and the pooled lower phases dried completely under nitrogen after which 750 µl of chloroform:methanol (1:2) was added and the sample moved to 1.5 ml chromatography vials (Waters, Milford, Massachusetts) and stored for a maximum of three days at -20°C until HPTLC analysis.

Silica gel HPTLC 60 F_{254} plates (Merck, Darmstadt, Germany) were cleaned prior to analyses with chloroform:methanol:acetic acid:water (25:17.5:3.8:1.75) and stored in a desiccator. Samples were removed from the freezer, and dried under nitrogen. Then 500 µl of chloroform:methanol (1:2) was added, the vial vortexed, and 5 µl of sample was applied on a silica plate with a Camag Automatic TLC Sampler 4 (Camag, Muttenz, Switzerland). On each plate a range of neutral lipid standards of known concentration were also sprayed (Supplement Table S2). The plate was placed in a horizontal through chamber (Camag, Horizontal Developing Chamber 2) and the neutral lipids were driven with hexane:ether:acetic acid:water (26:6:0.4:0.1). The plate was developed by first dipping for 3 sec (Camag, Chromatogram Immersion Device III) in a 3% copper sulfate 8% phosphoric acid solution, dried, and finally heated using a plate heater until lipid bands were clearly visible (about 3 minutes at 180°C). After development, plates were scanned with a Camag TLC plate scanner 3 at 254 nm using a deuterium lamp with a slit dimension of 6 × 0.45 mm and analyzed with the Win-CATS 1.1.3.0 software.

Resulting chromatographic peaks that represented the four major neutral lipid classes (diacylglycerols [DAG], triacylglycerols [TAG], cholesterol and cholesterol esters [CE]) were identified by comparing their retention times against those of known standards (Table S2). Then the peak areas were integrated and the amount of lipid per lipid class (in pmol) in the sample counted using the formula: $pmol_{sample} = (Area_{sample}/Area_{standard}) \times pmol_{standard}$. The relative contents of the lipid classes (molar percentage) were calculated by dividing the pmol content of a specific lipid class with the sum of pmol contents of all neutral lipid classes. In addition, the total neutral lipid contents (nmol per abdomen) were calculated as a sum of the contents of the individual

lipid classes. For the downstream statistical analyses the neutral lipid contents were regressed against abdomen weight and standardized residuals (i.e. mass-corrected storage lipid amount).

Measuring wing color

In the cold treatment we observed intermediate wing color phenotypes that varied from a pale to buttery yellow. Such intermediate phenotypes have also been observed in *C. eurytheme*. These individuals are predicted to have an Alba genotype because xanthopterin, the most abundant colored pigment in *C. eurytheme* wings, does not reach 50% of that seen in orange females (Hoffmann, 1974). We measured the wing color of dissected forewings from individuals used in the lipid analyses via digital photography. To decrease variation between images, photographs were taken by placing the wings on a solid blue background under a mounted Nikon D5100 with an AF-5 Micro NIKKOR 60 mm lens in a completely dark room. A Sigma EM-140DG ring flash was the sole light source for each photograph and the distance between the camera and wings was the same for each image. The camera setting was "automatic". Images were converted to JPEG format and an Auto White Balance/ Auto levels function from the GIMP (GNU Image Manipulation Program) software was applied to the images (in-house script, available upon request). Images were then imported to ImageJ v.1.49 (Schneider *et al.*, 2012). The "adjust-color threshold" tool was used to select only non-melanic portions of the wing for color analysis (Figure S1). The Red-Green-Blue (RGB) standard values (from 0-255) were calculated for the selected pixels and the mean red value for the selected area was recorded (where 0 is no red and 255 the most red). We hereby refer to this measure as "mean red". We also wished to quantify wing color for individuals on which we measured the other life history traits, however, many of the individuals we used for these data (eg. MR and development times) eclosed within the syringes used for the respirometry. As a result their wings did not expand properly and could not be photographed. Instead we classified them as Alba, yellow, or orange upon eclosion.

Statistics

All statistics were performed in R version 3.2.3 (R Core Team, 2015). Two factor ANOVAs were used to test the effects of morph and temperature on the measured life history traits and lipids classes (Table 1). Only females (Alba and orange) were used for these analyses. Mixed

models were fit to the respirometry data using the *lmer* function in the lme4 package (Table S3) (Bates *et al*., 2015). The response variables in the models were weight corrected MR, which was log-transformed to reduce heteroscedacity, and RQ. We first fit full models for both response variables; the fixed effects were morph (as a factor), treatment (as a factor), and the interaction between morph and treatment. To account for repeated measures on the same individuals a random intercept and random temporal slope were included in the model for individual and day measured. To identify the best-fit model we individually excluded the fixed effect terms and compared the AIC. The model with the lowest AIC score was considered the best fit. If the difference in AIC was less than 2 the simpler model was considered the better fit (Table S3). We tested the hypothesis that wing color varies inversely with abdominal lipid stores (i.e females with more color on their wings should have smaller lipid stores) by regressing mean red on the various lipid class measurements using the lm function. Finally to explore the effect of the intermediate yellow morphs on other life history traits, we applied morph (Alba, yellow, orange) as a factor in a one-way ANOVA for life history trait where Alba and orange differed (larval development rate).

Results

Effect of morph on life history traits

A two-way ANOVA revealed a trend for an interaction between morph and temperature on the larval development rate ($F_{1,70} = 2.9$, $p = 0.09$) (Table 1, Figure 2). Tukey's HSD post hoc testing revealed a trend for Alba females to develop slower than orange within the cold treatment ($p = 0.08$), while in the hot treatment there was no difference between morphs ($p = 0.99$). Temperature had a significant effect on the pupal development rate ($F_{1,92} = 9022.45$, $p < 0.0001$), adult wet weight ($F_{1,167} = 110.57$, $p < 0.0001$), and abdomen to thorax ratio ($F_{1,167} = 42.21$, $p < 0.0001$) but no significant effect of morph or a significant interaction between the factors was observed (Table 1, Figure 2). Respirometry measurements were conducted during the pupal phase only. The best-fit model for the log(weight corrected MR) included morph, treatment, and the interaction between them (Table S3). The best-fit model for RQ only included the random effects, individual and day measured (Table S3).

Effect of morph on abdominal lipid contents

A two-way ANOVA revealed a significant interaction between morph and temperature on the weight corrected cholesterol content ($F_{1,53}$ = 14.85, p = 0.0003) (Table 1, Figure 3). Tukey's HSD post hoc testing revealed orange females exhibited significantly higher cholesterol contents in the hot treatment (p = 0.00001), while no difference between morphs was observed in the cold treatment (p = 1.0). There was also a significant interaction between morph and temperature on the weight corrected CE stores ($F_{1,53}$ = 13.39, p = 0.0005) (Table 1, Figure 3). Again Tukey's HSD post hoc testing found orange females exhibited significantly larger stores than Alba in the hot treatment (p = 0.03), but no difference between morphs in the cold treatment (p = 0.11). We observed a significant effect of temperature ($F_{1,53}$ = 5.02, p = 0.03) and morph ($F_{1,53}$ = 5.67, p = 0.02) on the weight corrected DAG stores and a trend for an interaction between main effects ($F_{1,53}$ = 3.61, p = 0.06) (Table 1, Figure 3). Post hoc testing revealed that Alba females exhibited significantly larger DAG stores in the cold treatment (p = 0.02), but found no difference in the hot (p = 0.99). Finally we observed a significant effect of morph ($F_{1,53}$ = 4.17, p = 0.05) and treatment ($F_{1,53}$ = 13.44, p = 0.0006) on the weight corrected TAG stores but no significant interaction (p = 0.42). Post hoc testing found no significant differences between morphs within each treatment, though on average Alba females exhibited larger TAG stores than orange within both treatments.

Wing color variation in cold treatment

We observed variation in wing color within the cold treatment that was not seen in the hot, some individuals exhibited varying shades of yellow rather than white or orange (Figure 3C). We used digital photography to quantify wing color by measuring the average red (from the RGB score) for pixels within a selected area of the photo (i.e. mean red). The mobilized lipids, CE ($F_{1,29}$ = 7.21, p = 0.012; weight corrected CE = 0.02 – 0.0005 x mean red) and DAG ($F_{1,29}$ = 11.44, p = 0.002; weight corrected DAG = 0.782 – 0.003 x mean red) decrease significantly with mean red, while TAG exhibited a similar, albeit weaker trend ($F_{1,29}$ = 4.2, p = 0.05; weight corrected TAG = 0.522 – 0.002 x mean red) (Figure 3). There was no significant relationship between cholesterols and mean red ($F_{1,29}$ = 0.003, p = 0.954) (Figure 3). Interestingly, we observed that it is not only Alba females that have significantly more color in the cold treatment ($t_{23.57}$ = 5.34, p < 0.0001, hot

Alba mean 166.58, cold Alba mean 187.12), orange females also exhibited a significant increase in mean red (i.e. were more orange) from the hot to the cold treatment ($t_{17.26} = 4.29$, $p < 0.001$, hot orange mean 218.67 cold orange mean 228.84). In contrast, males show no such shift in wing color between temperature treatments ($t_{11.99} = 0.16$, $p = 0.878$, hot male mean red 231.33 cold male mean 231.7).

We did not have high quality wings for the individuals on which we measured the other life history traits, as many emerged within the respirometry chambers. Therefore we could not use photography to measure mean red, but instead classified them into three morphs Alba, yellow, and orange upon eclosion. Interestingly, a one-way ANOVA on larval development rate ($F_{2,41} = 7.71$, $p = 0.001$), the life history trait where Alba and orange females exhibited differences, revealed that the yellow individuals drive this difference. A post hoc Tukey-test revealed that yellow morphs developed significantly slower than orange ($p = 0.001$) in the larval stage and showed a trend for being slower than Alba ($p = 0.086$), while Alba and orange did not significantly differ ($p = 0.962$) (Figure 4).

Discussion

Here we have explored physiological differences associated with a female limited ALHS and the potential genotype x environment interactions that may contribute to its evolution and maintenance. Similarities between our findings and those from other *Colias* species suggest a shared evolutionary origin and associated trade-offs between Old and New World species and likely the genus *Colias,* since the Old and New World split is close to the base of the genus (Brunton, 1998; Pollock *et al.*, 1998; Wheat & Watt, 2008), wherein temperature plays a significant role in the selection dynamics acting upon the Alba ALHS (Graham *et al*.,1980; Nielsen & Watt, 1998).

The first line of evidence suggesting Alba arises via the same mechanism is the presence of yellow females in the cold treatment (Figure 3C) as cold temperatures also induce a yellow phenotype in *C. eurytheme* (Hoffmann, 1974). Yellow individuals are predicted to have an Alba genotype because the levels of xanthopterin (yellow), the most abundant colored pigment in orange *C. eurytheme* wings, does not reach 50% of that seen in orange females (Hoffmann,

1974). The presence of intermediate color phenotypes indicates a plastic response to temperature, likely due to regulatory loci or perhaps due to temperature's effect on the pteridine biosynthesis pathway. In orange *C. eurytheme* pupa, the rate of pteridine synthesis begins slowly and increases as development progresses (Watt 1973). As the synthesis rate increases, so does the concentration of 7,8-dihydroxanthopterin, the precursor to leucopterin (colorless) and xanthopterin (yellow) (Watt 1973). At increased concentrations 7,8-dihydroxanthopterin, and to some extent leucopterin, negatively inhibit xanthine dehydrogenase (XDH), the enzyme that converts xanthopterin to leucopterin, and colored pteridines begin to accumulate. Watt (1973) predicts that in Alba females a reduction in the amount of precursors entering the pteridine synthesis pathway could result in 7,8-dihydroxanthopterin never accumulating at a high enough level to fully inhibit XDH, thereby preventing the accumulation of the colored pteridines and giving rise to the white wing phenotype. However if the cold treatment increased the rate at which precursors entered the pathway such that 7,8-dihydroxanthopterin could accumulate faster and inhibit XDH either more effectively or earlier in development, this could potentially lead to the accumulation of xanthopterin (yellow) in Alba individuals. This hypothesis could also explain the increase in mean red observed in orange females in the cold treatment.

Results from our lipid analysis also suggest a shared mechanism for Alba within the genus. First Alba *C. croceus* females have larger abdominal lipid stores than orange females, though the difference is only significant in the cold treatment. These results are consistent with previous works that found the Alba females of *C. alexandra* and *C. scudderi* had larger fat bodies than orange and that in *C. eurytheme* the Alba trade-off was more pronounced under colder (i.e. more stressful) environments (Graham et al., 1980).

Graham et al. (1980) found *C. eurytheme* Alba pupa develop 4% faster than orange. A similar result was not observed in the present study, and there are several possible explanations for this unexpected result. Food stress, caused by increased larval density, may induce the development rate differences. The larvae in the previous work were reared in a large colony (Graham *et al.*, 1980) and the author (A. Woronik) has observed that Alba females of *C. croceus* generally emerge before orange during other experiments where larvae were reared in groups, rather than individually, as in this study. As such, the rearing on high quality host plant would only result in feeding stress under higher densities. This hypothesis is supported by field data, with Alba

frequency and fecundity higher in *Colias* species that feed on nutrient poor host plant, suggesting that Alba has increased fitness in nutrient poor habitats (Nielsen & Watt, 1998).

Our work also suggests that similar to other *Colias* species (e.g. *C. scudderi* and *C. Alexandra;* Nielsen & Watt, 1998), temperature plays a significant role in the selection dynamics acting on Alba in *C. croceus.* In the hot treatment orange females had significantly larger cholesterol and cholesterol ester contents than Alba, while there were no differences in the other lipid classes. Usually in animal tissues, the alcoholic (unesterified) cholesterol is located in cellular membranes and not in the storage lipid droplets. Thus our data suggests that the orange morphs have higher membrane cholesterol content, which could relate to maintenance of membrane integrity at elevated temperatures (Crockett, 1998). In line with this is the ability of the orange morph to maintain high MR towards the end of the 6-day inspection period when kept in the hot treatment (Figure 5). In the cold treatment, Alba females had significantly larger neutral lipid stores residing in storage droplets, i.e. CE and DAG (Figure 3) (Arrese, 2001; Arrese, 2010; Fujimoto & Parton, 2011). Increased content of neutral lipids, which can be mobilized for energy, is indicative of a capacity of growth and reproduction. These results are consistent with previous work that found Alba females had higher fecundity than orange females in *C. scudderi*, a species that inhabits a colder environment and feeds on a low nitrogen content host plant (Nielsen & Watt, 1998). In comparison orange females of *C. alexandra*, a species that flies at warmer temperatures and feeds on a nitrogen rich host plant, had a higher fecundity than Alba (Nielsen & Watt, 1998). Nielsen and Watt (1998) hypothesized that environmental heterogeneity in temperature could create distinct selection regimes that lead to varying frequencies of Alba across populations. Our results suggest a similar selective mechanism could be acting on these strong flying butterflies. Quantifying the Alba frequency and temperature regimes across *C. croceus* populations could add insight to this hypothesis and will be an important future research avenue to pursue.

We also found evidence indicating that the intermediate color morphs (yellow) may have decreased fitness. Yellow females developed significantly slower in the larval stage than orange and there was a trend for them being slower than Alba, while the Alba and orange females did not significantly differ (Figure 4). An increased cost of development for the yellow phenotype may manifest as disruptive selection in the field, thereby favoring the maintenance of the two

discrete morphs that lack this trade-off (Futuyma, 2013). This hypothesis is supported by field observations, as the yellow morphs observed in wild are extremely rare, at least in Spanish *C. croceus* populations (personal observations of C. Stefanescu).

The aim of our work was to add insight into the evolution of, and the selective mechanisms acting on, the Alba ALHS by investigating the potential trade-offs in life history and physiological traits between morphs at different temperatures in *Colias croceus*. We find evidence that the Alba mechanism is likely conserved between the New and Old World species indicating either a single evolutionary origin of Alba or repeated co-option of the same mechanism. Additionally, similar to the New World species, temperature seems to play an important role in the selection dynamics acting on Alba. Alba females seemed to have an advantage over orange in energy metabolism related physiological traits that could be related to flight performance, growth and fecundity when reared at low temperatures. Conversely, orange females seemed to exhibit some advantages over Alba in warm. Also, under cold conditions, an additional phenotype was observed that is rare in wild *C. croceus* populations - yellow females. These individuals of intermediate phenotypes, between the classic ALHS phenotypes of Alba and orange, have potentially lower fitness, given their increased larval development time. Disruptive selection on the intermediate phenotype, combined with morph specific advantages based on environmental temperature, could maintain the two morphs in the field.

Author Contributions

AW conducted the fieldwork, butterfly rearings, respirometry measurements, and dissections and wrote the manuscript with contributions from coauthors. CS conducted fieldwork. AW and PL analyzed the data. PL contributed to the respirometry measurements and conducted the lipid analysis with RK. PL and CWW supervised the work.

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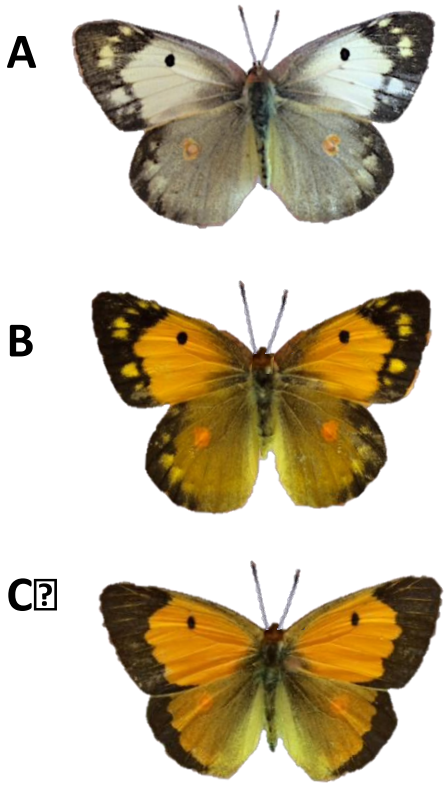

Figure 1 Color variation in *Colias croceus*. A) Alba female B) orange female C) male. Photo credit A. Woronik

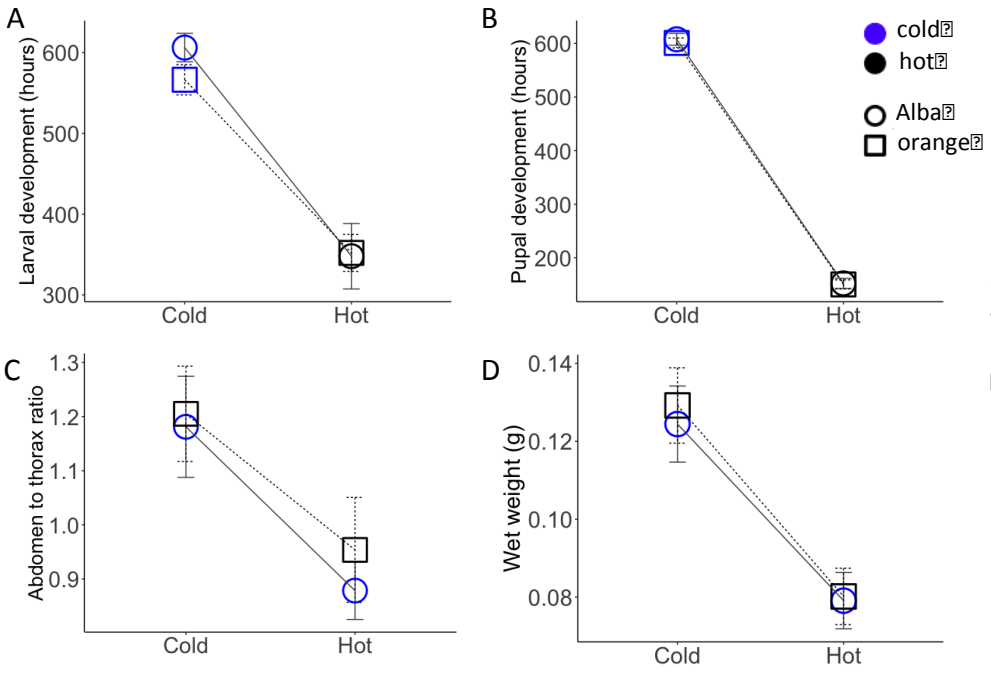

Figure 2 Interaction plots for the major life history traits in *Colias croceus.* Error bars represent the 95% confidence intervals. Circles are Alba females, while squares are orange females. Blue shapes are the cold treatment while black are the hot. A) Larval development time in hours B) Pupal development time in hours C) Abdomen to thorax ratio D) Wet weight in grams.

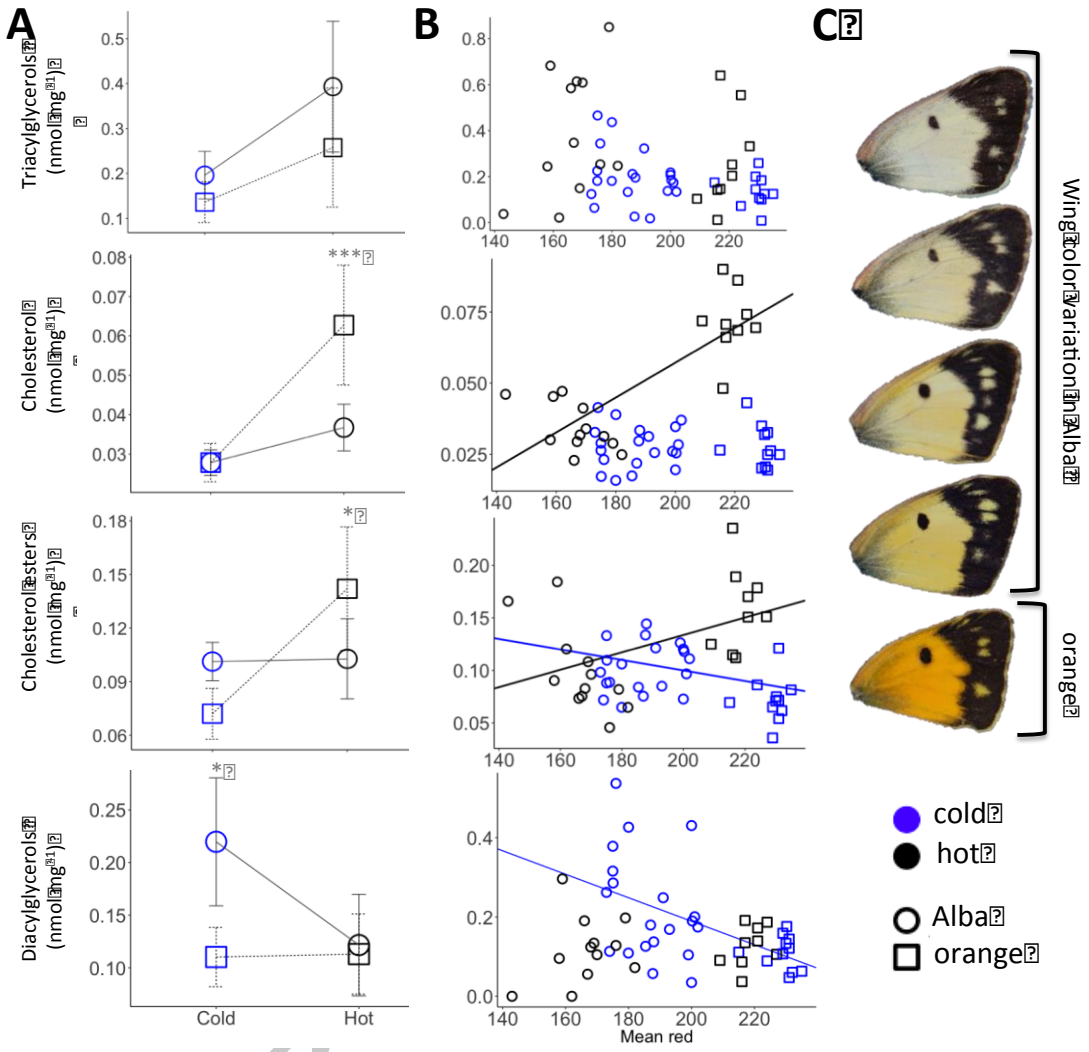

Figure 3 Analyses of major lipid classes, triacylglycerols (TAG), cholesterol, cholesterol esters (CE), and diacylglycerols (DAG) from abdomens of Alba females and orange females of *Colias croceus*. Alba females are indicated as circles, while orange females are squares. The cold treatment is colored blue, while the hot is black. A) Interaction plots illustrating lipid differences between female morphs within temperature treatments. Error bars are the 95% confidence intervals. Significance stars are indicated as follows 0 '***' 0.001 '**' 0.01 '*' 0.05. B) Regression of the mean red (wing color) and the abdominal stores of neutral lipids. C) Wing color variation in Alba individuals reared in the cold treatment compared to an orange individual reared in the cold treatment.

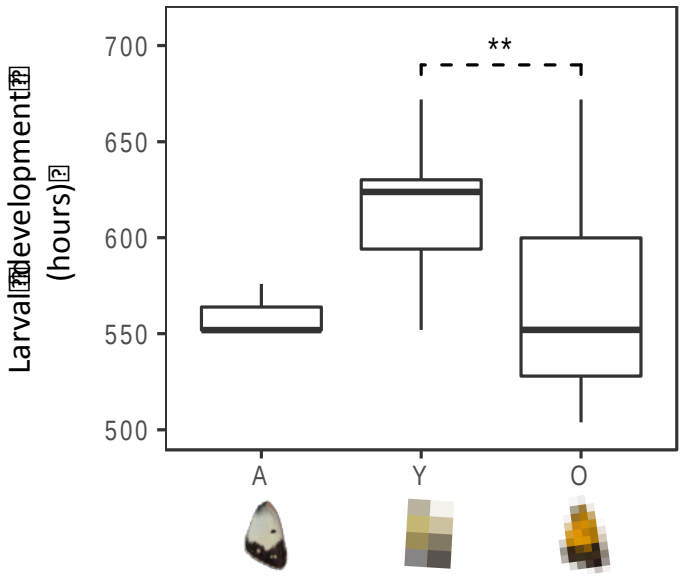

Figure 4 A boxplot comparing the larval development rate in the cold treatment for *Colias croceus* with females classified into three morphs: A is Alba, Y is yellow, and O is orange. Significance stars are indicated as follows 0 '***' 0.001 '**' 0.01 '*' 0.05.

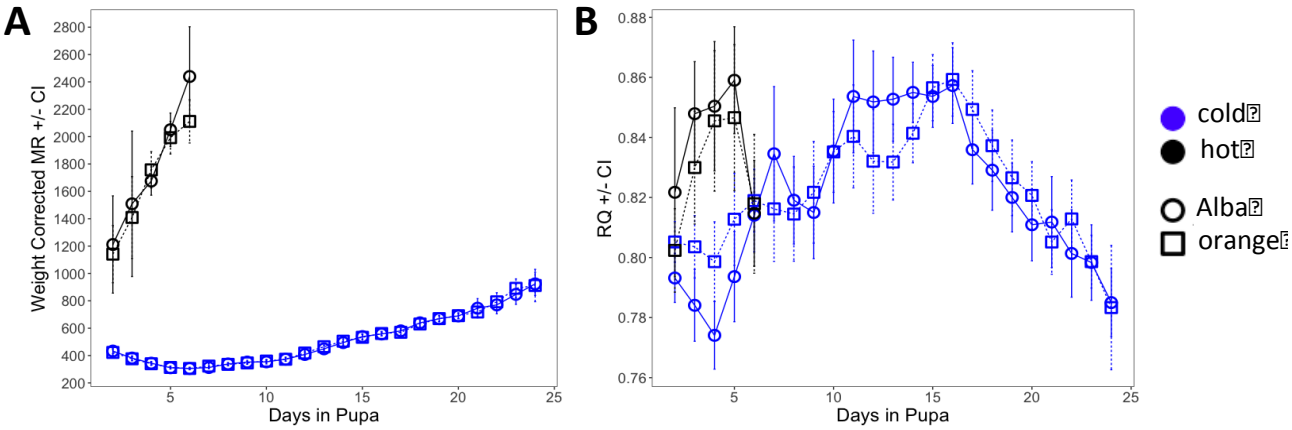

Figure 5 Measuring metabolic rate (MR) and respiratory quotient (RQ) during pupal development in *Colias croceus*. Error bars represent the 95% confidence interval. Circles represent Alba females, while the squares represent orange. Blue coloration indicates the cold treatment, while black represents the hot. A) Weight corrected MR over the course of pupal development. B) RQ over the course of pupal development.

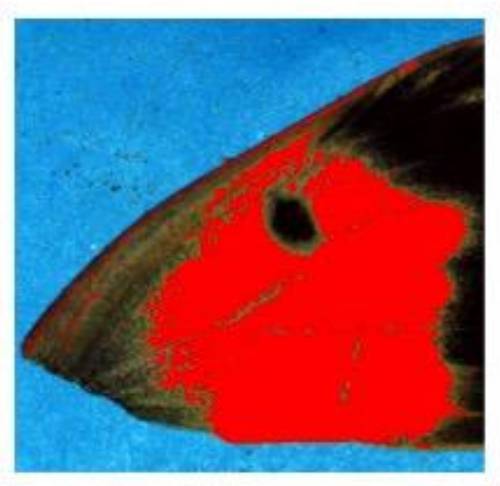

Figure S1 An example of the area selected of the wing image to measure mean red. Red region is considered when calculating 'mean red'

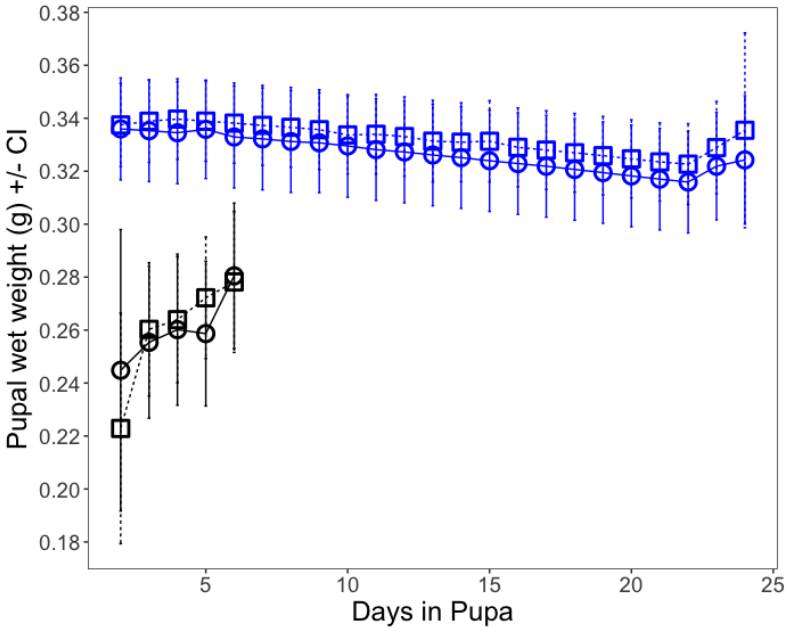

Figure S2 Pupal weight in grams over the course of development for *Colias croceus*. Alba females are indicated as circles, while orange females are squares. The cold treatment is colored blue, while the hot is black. Error bars represent the 95% confidence intervals.

Table 1 Results of 2 factor ANOVAs. Only females, Alba and orange, were used in these analyses. Where DAG is diacylglycerols, TAG is triacylglycerols, and CE is cholesterol esters.

Response Variable	Explanatory Variable	*F*	df	*P*
Larval development rate (hours)	**Temperature**	**342.25**	**1,70**	**<0.0001**
	Morph	3.1	1,70	0.08
	Temperature x Morph	2.9	1,70	0.09
Pupal development rate (hours)	**Temperature**	**9022.45**	**1,92**	**<0.0001**
	Morph	0.93	1,92	0.34
	Temperature x Morph	0.25	1,92	0.61
Adult wet weight (g)	**Temperature**	**110.57**	**1,167**	**<0.0001**
	Morph	0.51	1,167	0.48
	Temperature x Morph	0.17	1,167	0.68
Abdomen to thorax ratio	**Temperature**	**42.21**	**1,167**	**<0.0001**
	Morph	1.18	1,167	0.28
	Temperature x Morph	0.35	1,167	0.55
Pupal developmental budget (J)	**Temperature**	**5096.4**	**1,84**	**<0.0001**
	Morph	**6.0**	**1,84**	**0.01**
	Temperature x Morph	1.6	1,84	>0.05
Cholesterol (nmol/mg $^{-1}$)	**Temperature**	**33.76**	**1,53**	**<0.0001**
	Morph	**12.67**	**1,53**	**0.0008**
	Temperature x Morph	**14.85**	**1,53**	**0.0003**
CE (nmol/mg $^{-1}$)	**Temperature**	**9.76**	**1,53**	**0.003**
	Morph	0.06	1,53	0.80
	Temperature x Morph	**13.39**	**1,53**	**0.0005**
DAG (nmol/mg $^{-1}$)	**Temperature**	**5.02**	**1,53**	**0.03**
	Morph	**5.67**	**1,53**	**0.02**
	Temperature x Morph	3.61	1,53	0.06
TAG (nmol/mg $^{-1}$)	**Temperature**	**13.44**	**1,53**	**0.0006**
	Morph	**4.17**	**1,53**	**0.05**
	Temperature x Morph	0.66	1,53	0.42

Table S1 Sample sizes for the respirometry experiment split by treatment, day of pupation, and morph.

Treatment	Days of pupation	Morph	N
Cold	2	Alba	22
		orange	27
	3	Alba	22
		orange	28
	4	Alba	22
		orange	29
	5	Alba	22
		orange	29
	6	Alba	22
		orange	29
	7	Alba	22
		orange	29
	8	Alba	22
		orange	29
	9	Alba	22
		orange	29
	10	Alba	22
		orange	29
	11	Alba	22
		orange	29
	12	Alba	22
		orange	29
	13	Alba	22
		orange	29
	14	Alba	22
		orange	29
	15	Alba	22
		orange	29
	16	Alba	22
		orange	29
	17	Alba	22
		orange	29
	18	Alba	22
		orange	29
	19	Alba	22
		orange	29
	20	Alba	22
		orange	29
	21	Alba	22
		orange	29
	22	Alba	22
		orange	29

	23	Alba	18
		orange	22
	24	Alba	10
		orange	9
Hot	2	Alba	7
		orange	6
	3	Alba	23
		orange	18
	4	Alba	24
		orange	20
	5	Alba	25
		orange	17
	6	Alba	19
		orange	13

Table S2 Standards used for HPTLC analysis.

Standard	Lipid representative	MW (g/mol)	Concentration, µM	Amount on plate, pmol (in 20 µl)
DAG	Glycerol dilaurate	456.7	22	420
TAG	Glycerol trioleate	885.4	50	1000
cholesterol	Cholesterol	386.7	50	1000
CE	Cholesterol linoleate	649.1	50	1000

Table S3 Model selection for generalized linear models on the log(weight corrected metabolic rate (MR)) and respiratory quotient (RQ) data from the full to null models. Model with the lowest AIC was considered the best fit, when the AIC difference was less than two the simpler model was chosen as the best fit. Best fit models are in bold. "Int" is the interaction between fixed effects. Males were excluded.

Response variable	Model	AIC	df
log(weight corrected MR)	**morph+treatment+int+(day measured| individual)**	**17273**	**8**
log(weight corrected MR)	morph+treatment+(day measured| individual)	17282	7
log(weight corrected MR)	morph+(day measured| individual)	17546	6
log(weight corrected MR)	treatment+(day measured| individual)	17290	6
log(weight corrected MR)	(day measured| individual)	17558	5
RQ	morph+treatment+int+(day measured| individual)	-4699	8
RQ	morph+treatment+(day measured| individual)	-4707	7
RQ	morph+(day measured| individual)	-4708	6
RQ	treatment+(day measured| individual)	-4718	6
RQ	**(day measured| individual)**	**-4719**	**5**

Table S4 Mean and standard deviations of traits measured, split by treatment, sex, and morph.

Trait	Treatment	N	Mean	SD
Larval development rate (hours)	**Hot**			
Males		15	324.76	45.21
Females (all)		30	349.54	66.25
Alba females		18	347.90	81.51
Orange females		12	351.99	35.94
Larval development rate (hours)	**Cold**			
Males		19	535.52	24.07
Females (all)		44	583.56	45.90
Alba females		19	606.19	36.50
Orange females		25	566.36	45.41
Pupal development rate (hours)	**Hot**			
Males		24	151.02	32.03
Females (all)		45	151.51	20.96
Alba females		26	152.02	32.03
Orange females		19	150.34	17.54
Pupal development rate (hours)	**Cold**			
Males		21	612.66	29.88
Females (all)		51	603.82	24.6
Alba females		22	607.71	24.96
Orange females		29	600.88	24.34
Pupal development budget (kJ)	**Hot**			
Males		23	1072.23	120.67
Females (all)		37	986.12	141.78
Alba females		21	1001.64	157.62
Orange females		16	965.75	119.75
Pupal development budget (kJ)	**Cold**			
Males		20	3748.89	180.92
Females (all)		51	3600.41	195.46
Alba females		22	3673.52	215.59
Orange females		29	3544.95	161.19
Adult wet weight (g)	**Hot**			
Males		34	0.074	0.021
Females (all)		75	0.080	0.022
Alba females		41	0.080	0.023
Orange females		34	0.080	0.021
Adult wet weight (g)	**Cold**			
Males		89	0.106	0.024
Females (all)		96	0.127	0.033
Alba females		49	0.124	0.034
Orange females		47	0.129	0.033
Abdomen to thorax ratio	**Hot**			
Males		35	0.831	0.358
Females (all)		75	0.913	0.227
Alba females		41	0.878	0.169
Orange females		34	0.954	0.278

Abdomen to thorax ratio	**Cold**			
Males		89	0.955	0.280
Females (all)		96	1.193	0.312
Alba females		49	1.181	0.326
Orange females		47	1.205	0.299
Diacylglycerols (nmol/mg $^{-1}$)	**Hot**			
Males		12	0.082	0.094
Females (all)		25	0.118	0.072
Alba females		14	0.122	0.084
Orange females		11	0.113	0.057
Diacylglycerols (nmol/mg $^{-1}$)	**Cold**			
Males		11	0.038	0.064
Females (all)		32	0.182	0.122
Alba females		21	0.220	0.134
Orange females		11	0.110	0.042
Triacylglycerols (nmol/mg $^{-1}$)	**Hot**			
Males		12	0.799	0.589
Females (all)		25	0.334	0.235
Alba females		14	0.393	0.252
Orange females		11	0.258	0.197
Triacylglycerols (nmol/mg $^{-1}$)	**Cold**			
Males		11	0.083	0.126
Females (all)		32	0.176	0.105
Alba females		14	0.196	0.116
Orange females		11	0.136	0.068
Cholesterol (nmol/mg $^{-1}$)	**Hot**			
Males		12	0.040	0.022
Females (all)		25	0.048	0.021
Alba females		14	0.037	0.010
Orange females		11	0.063	0.023
Cholesterol (nmol/mg $^{-1}$)	**Cold**			
Males		11	0.026	0.006
Females (all)		32	0.028	0.007
Alba females		21	0.028	0.007
Orange females		11	0.028	0.007
Cholesterol ester (nmol/mg $^{-1}$)	**Hot**			
Males		12	0.444	0.298
Females (all)		25	0.120	0.048
Alba females		14	0.103	0.039
Orange females		11	0.142	0.051
Cholesterol esters (nmol/mg $^{-1}$)	**Cold**			
Males		11	0.046	0.014
Females (all)		32	0.091	0.026
Alba females		21	0.101	0.024
Orange females		11	0.072	0.021
Mean red	**Hot**			
Males		6	231.333	4.227

Females (all)		21	-	-
Alba females		12	166.583	10.483
Orange females		9	218.667	5.268
Mean red	**Cold**			
Males		10	231.7	4.9
Females (all)		31	-	-
Alba females		20	187.12	10.634
Orange females		11	228.839	5.294